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The anti-tumor effect of cross-reacting material 197, an inhibitor of heparin-binding EGF-like growth factor, in human resistant ovarian cancer

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ABSTRACT

Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is a promising target for ovarian cancer therapy. Cross-reacting material 197 (CRM197), a specific HB-EGF inhibitor, has been proven to represent possible chemotherapeutic agent for ovarian cancer. However, the effect of CRM197 on the resistant ovarian carcinoma cells has not been sufficiently elucidated. Here, we found that HB-EGF was over-expressed in a paclitaxel-resistant human ovarian carcinoma cell line (A2780/Taxol) and a cisplatin-resistant cell line (A2780/CDDP), as well as the xenograft mouse tissue samples with these cells. To investigate the possible significance of the HB-EGF over-expression in A2780/Taxol and A2780/CDDP cells, we inhibited HB-EGF expression by CRM197 to investigate the effect of CRM197 treatment on these cells. We observed that CRM197 significantly induced anti-proliferative activity in a dose-dependent manner with the cell-cycle arrest at the G0/G1 phase and enhanced apoptosis in A2780/Taxol and A2780/CDDP cells. The sensitive ovarian carcinoma parental cell line (A2780), A2780/Taxol and A2780/CDDP cells formed tumors in nude mice, and enhanced tumorigenicity was observed in drug-resistant tumors. Furthermore, we observed that CRM197 significantly suppressed the growth of drug-resistant ovarian cancer xenografts *in vivo* ($p < 0.001$). These results suggest that CRM197 as an HB-EGF-targeted agent has potent anti-tumor activity in paclitaxel- and cisplatin-resistant ovarian cancer which over-express HB-EGF.

1. Introduction

Ovarian cancer is the leading lethal cause among all gynecological malignancies. More than 80% of patients respond to first-line chemotherapy with platinum and taxane. However, nearly all the patients relapse and become refractory to traditional chemotherapeutics. Patients who relapse, and those who do not initially respond to chemotherapy, are thought to carry hidden drug-resistant cells, leading to tumor relapse and lethality [1]. Therefore, a effective novel therapy for drug-resistant ovarian cancer at the molecular level is eagerly awaited.

Heparin-binding epidermal growth factor-like growth factor (HB-EGF), an epidermal growth factor receptor (EGFG) ligand, is synthesized as a membrane-anchored protein (pro-HB-EGF), which is cleaved at the cell surface by a protease to release a soluble N-terminal ectodomain (s-HB-EGF) via a mechanism referred to as ectodomain shedding [2]. S-HB-EGF, a potent mitogen and chemoattractant, drives signal-transduction cascades through activating EGFR or erythroblastic leukemia viral oncogene homolog 4 (ErbB4), which have critical roles in diverse pathophysiological processes [3–5]. Meanwhile, the C-terminal domain of HB-EGF (after ectodomain shedding) translocates to the nucleus and modulates the cell cycle and cell proliferation [6]. HB-EGF participates in a variety of pathophysiological processes and is over-expressed in many human malignancies, such as bladder carcinoma [7], gastric cancer [8], head and neck squamous cell carcinoma [9] and ovarian cancer.

So far, the role of HB-EGF over-expression in ovarian cancer cells has been increasingly reported [10]. The tumors and ascitic fluid obtained from patients with ovarian cancer express higher levels of HB-EGF than the normal ovaries and ovarian cysts. Meanwhile, there were large differences in expression between HB-EGF and the other six EGFR ligands [11]. HB-EGF has been proven to play a pivotal role in tumorigenicity [11], proliferation [12], metastasis and angiogenesis [13], making HB-EGF a promising therapeutic target for ovarian carcinoma. HB-EGF is over-expressed in 5-fluorouracil (5-FU)- and cisplatin (CDDP)-resistant cells and is regarded as a chemoresistance-related gene in gastric cancer, colon cancer and et al. [14–16]. However, the expression level of HB-EGF and its precise role in drug-resistant ovarian cancer remains unclear.

Cross-reacting material 197 (CRM197), a non-toxic mutant of diphtheria toxin, is a strong specific HB-EGF inhibitor [17].

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CRM197 binds to the sHB-EGF, as well as to proHB-EGF, and inhibits the mitogenic action of HB-EGF by inhibiting its binding to ErbB receptors [18]. Currently, a phase I study on CRM197 is being conducted for patients with advanced ovarian cancer at Fukuoka University, Japan [10]. CRM197 has been proven to represent possible chemotherapeutic agent for ovarian cancer [10,19,20]. However, its effect on resistant ovarian cancer cells has not been sufficiently elucidated.

The current study examined the inhibitory effects of CRM197 on resistant human ovarian cancer cells. We found that the levels of HB-EGF expression in paclitaxel- and cisplatin-resistant cells were higher than the levels in parental sensitive cells, and CRM197 treatment which inhibited HB-EGF expression could significantly suppress the tumorigenicity of resistant ovarian cancer *in vitro* and *in vivo*.

2. Materials and methods

2.1. Cell culture and CRM197 treatment

The cisplatin-resistant human ovarian cancer cell line (A2780/CDDP; catalog No. 93112517) was purchased from the European Collection of Cell Cultures (ECACC, UK) and cultured according to the ECACC recommendations. The sensitive human ovarian cancer parental (A2780) and the paclitaxel-resistant (A2780/Taxol) cell lines were gifts of Dr. Lan Xiao (Department of Gynecology and Obstetrics, the Third Affiliated Hospital of Sun Yan-sen University, Guangzhou, China) and were cultured as previously described [21]. For the CRM197 (Sigma–Aldrich, USA) treatment, the cells were seeded in 6-well plates at a densities of 5×10^4 cells/well and treated with CRM197 at the concentration of 100 μg/ml for 24 h.

2.2. Western blot

For HB-EGF, the total cell lysate was used, and Western blot analysis was performed as previously described. The proteins were separated by SDS–PAGE and transferred onto nitrocellulose membranes. The Enhanced Chemiluminescence (ECL) detection reagent (Amersham Biosciences, USA) was used to visualize the bands. Signal intensity was determined by Quantity One version 4.6.9 Windows.

2.3. Immunohistochemistry

The tissues from ovarian cancer xenograft model to core were based on review of the H&E slides. Immunohistochemistry was done using the HB-EGF mouse monoclonal antibody (Abcam PLC., UK) at a dilution of 1:100 and a histostain–streptavidin–peroxidase kit (Zhongshan Goldenbridge Biotechnology Co., Ltd., China). Immunoreactivity was scored by two investigators as follows: 0, undetectable; 1+, weakly positive; 2+, moderately positive; and 3+, intensely positive. HB-EGF immunoreactivity was not detectable (immunointensity score, 0) in normal nude mice tissue.

2.4. MTT cell viability assay

The drug-sensitive and -resistant cells were seeded at 10^4 cells/well in 96-well plates and incubated with varying concentrations of CRM197 (0, 0.01, 0.1, 1, 10 and 100 μg/ml) for 24 h. After 48 h incubation, 20 μl of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bro-mide (MTT) solution (5 mg/ml, Sigma–Aldrich) was added to each well of 96-well plates and incubated for another 4 h at 37 °C. The cells were then solubilized with dimethyl sulfoxide. The absorbance at 490 nm was measured using a Microplate Reader (ELX800, Bio-Tek).

2.5. Cell-cycle analysis

Cells with and without CRM197 treatment were collected and centrifuged at 1000 rpm, then washed twice with ice–cold PBS and fixed in ice–cold 70% ethanol overnight at 4 °C. Fixed cells were centrifuged at 1000 rpm and stained with 300 μl PI at 20 μg/ml by incubating at 37 °C for 20 min. The DNA content of cells was analyzed at a wavelength of 488 nm using a flow cytometer (FACScan Calibur, Becton Dickinson, USA) with Cell Quest software.

2.6. Caspase-3 activity assay

Caspase-3 activity assay was carried out according to instructions of the colorimetric activity assay kit (R&D Systems). Cells with or without CRM197 treatment were lysed and analyzed for total protein by the Bradford assay. Samples containing 20 μl total protein were assayed for caspase-3 activity with Ac-DEVD as a caspase-3-specific substrate. Absorbance was measured at 405 nm in a plate reader (ELX800, Bio-Tek).

2.7. Xenograft model

Ovarian cancer xenografts were established with 4-week-old female BALB/c nude mice (Vital River Laboratory, China). The A2780, A2780/Taxol and A2780/CDDP cells were trypsinized and respectively resuspended in PBS. The mixture containing 10^7 cells in a volume of 200 μl was subcutaneously injected into the flanks of female mice (10 mice/group). Tumor size was determined by multiplying by $0.5 \times \text{width}2 \times \text{length}$. For 4 weeks of CRM197 treatment, CRM197 dissolved in 200 μl PBS (1 mg/week) was injected intraperitoneally into tumor-bearing mice (5 mice/group) each week. All experimental use of animals complied with the guidelines of Animal Care of Haerbin Medical University.

2.8. Statistical analysis

The values shown are representative of triplicate data from Western blotting analyses and flow cytometry. Data are expressed as the mean ± SD from three independent experiments. Statistical significance ($P < 0.05$) was assessed by *t*-test (Sigmastat v. 3.5 software, USA).

3. Results

3.1. Enhanced expression of HB-EGF in drug-resistant human ovarian cancer cells and xenograft mice models

HB-EGF is the primary EGFR ligand altered in ovarian cancer. Its expression level in ovarian cancer is much higher than those of normal ovaries and ovarian cysts. We first determined HB-EGF expression in drug-resistant ovarian cancer cells by western blot analysis using drug-sensitive cells as a control (Fig. 1A, $p < 0.001$). The data showed that the A2780/Taxol and A2780/CDDP cells exhibited significant increases in HB-EGF expression compared with the A2780 cells. We also used immunohistochemical staining to examine the HB-EGF expression in xenograft mouse models established with A2780, A2780/Taxol and A2780/CDDP cells (10 mice/group). Consistent with the data *in vitro*, the immunointensity of the A2780/Taxol and A2780/CDDP tumors was 3+ and the immunointensity of the A2780 tumors was 2+ (Fig. 1B). Taken together, both *in vitro* and *in vivo* data indicate that HB-EGF expression in drug-resistant ovarian cancer is significantly elevated compared with drug-sensitive ovarian cancer, suggesting that HB-EGF

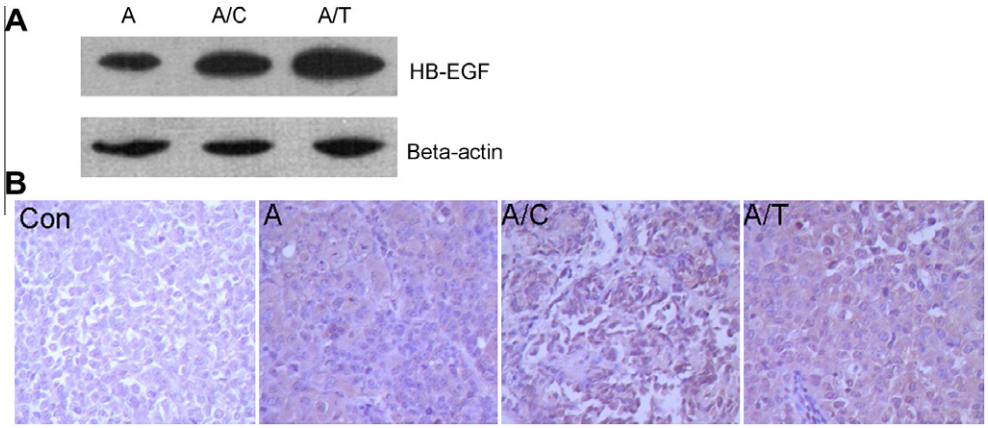

Fig. 1. Enhanced expression levels of HB-EGF in drug-resistant human ovarian cancer cell lines and xenograft mice models. (A) HB-EGF expression was assessed in the parental A2780, A2780/CDDP and A2780/Taxol cells by Western blotting using an anti-HB-EGF antibody. Beta-actin was used as a loading control. (B) Immunoreactivity of HB-EGF in the tissues from ovarian cancer xenograft models was examined by immunohistochemistry. The tissue samples with negative staining of HB-EGF were used as a control. The images of immunohistochemistry were taken under a light microscope (400×). Abbreviation used: Con, control; A, A2780; A/C, A2780/CDDP; A/T, A2780/Taxol.

may be associated with the chemoresistance of resistant ovarian cancer to paclitaxel and cisplatin therapies.

3.2. CRM197 suppresses cell proliferation and enhances apoptosis in the A2780/Taxol and A2780/CDDP cells

To examine the effect of CRM197 on A2780/Taxol and A2780/CDDP cells which over-express HB-EGF, cell viability was evaluated by MTT assay under varying concentrations of CRM197 treatment conditions. The data showed that CRM197 reduced cell viability rates in the concentration-dependent manner in drug -resistant cell lines (Fig. 2A, $p < 0.001$). We further evaluated the cell-cycle analysis and caspase-3 activity. As shown in Fig. 2B, CRM197 dramatically arrested drug-sensitive and -resistant cells at G0/G1 phase. After CRM197 treatment, A2780/Taxol and A2780/CDDP cells were accumulated in the G0/G1 phase by 47.04% and 37.78% increase respectively whereas A2780 cells increases G0/G1 cell-cycle arrest by 12.7%. Consistent with its effect on cell cycle, CRM197 treatment in A2780/Taxol and A2780/CDDP cells led to significant inhibition of the caspase-3 activity when compared to the A2780 cells (Fig. 2C). Taken together, *in vitro* data indicate that CRM197 potently induces G0/G1 cell-cycle arrest and apoptotic cell death in a dose-dependent manner in A2780/Taxol and A2780/CDDP cells.

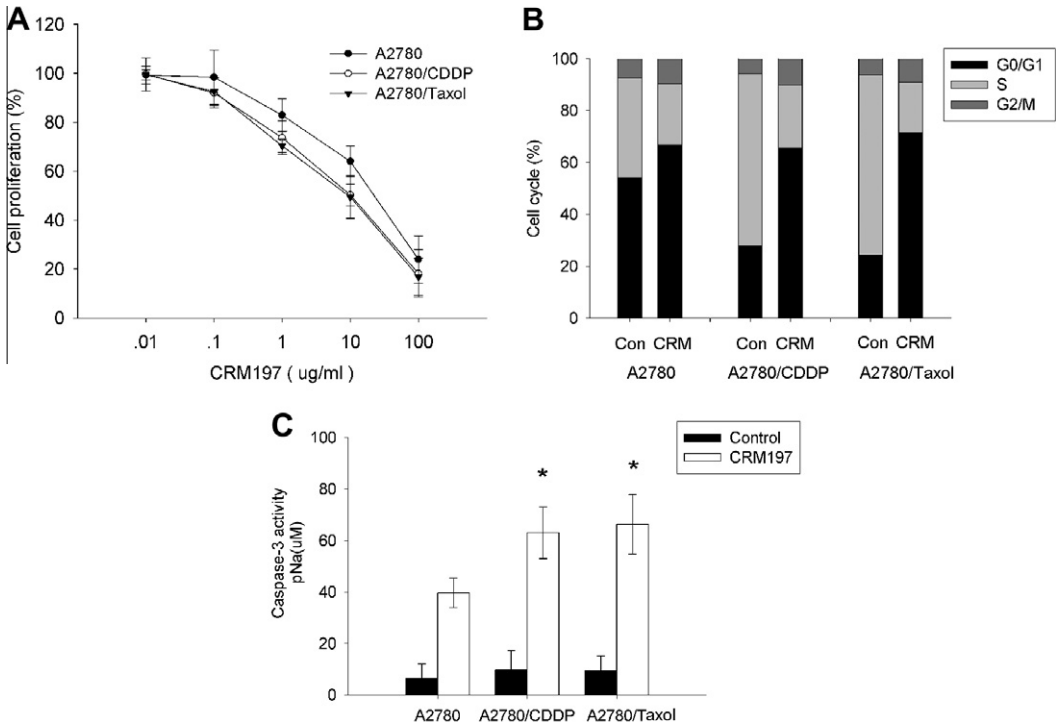

Fig. 2. CRM197 suppressed cell proliferation and enhanced apoptosis in the A2780/CDDP and A2780/Taxol cells. (A) MTT cell viability assay for A2780, A2780/CDDP and A2780/Taxol cells treated with varying concentrations of CRM197 (0, 0.01, 0.1, 1, 10 and 100 μg/ml) for 24 h. (B) Flow cytometry analyses of cell-cycle for A2780, A2780/CDDP and A2780/Taxol cells cultured in the absence (control) or presence of CRM197 at 100 μg/ml for 24 h. Data shown are representatives of three independent experiments. Abbreviation used: Con, control; CRM, CRM197. (C) caspase-3 enzymatic activity assay for the cells cultured alone (control) or treated with CRM197 at 100 μg/ml for 24 h. ∗, $P = 0.002$, versus the control group. The values represent the mean ± SD for one experiment performed in triplicate.

3.3. CRM197 suppresses the growth of drug-resistant ovarian cancer xenografts in vivo

We established xenograft mouse models with A2780, A2780/CDDP and A2780/Taxol cells. Two weeks after subcutaneous injection, enhanced tumorigenicity was observed in drug-resistant ovarian tumors. The A2780/Taxol and A2780/CDDP cells, which express higher levels of HB-EGF, formed larger tumors than the A2780 cells (Fig. 3D). Therefore, we hypothesize that HB-EGF may contribute to tumor formation of drug-resistant cancer.

To confirm our hypothesize, we examined the effect of CRM197 on tumor growth after 2 weeks of tumor growth. During 4 weeks of CRM197 treatment, tumor mass was measured every week. We found that tumor formation from three cell lines, especially drug-resistant cells, was effectively suppressed by CRM197 treatment (Fig. 3A–C). We observed more significant reductions in tumor size and mass of the A2780/CDDP and A2780/Taxol tumors than those of the A2780 tumors. After 4 weeks of CRM197 treatment, mice were sacrificed and the tumors were collected for weight measurement. Similarly, we found that tumor weight of the CRM197-treated groups was all significantly less than that of the untreated group (Fig. 3D). These results confirm that CRM197 effectively suppresses tumor formation of drug-resistant ovarian tumors compared with drug-sensitive tumors *in vivo*.

4. Discussion

In ovarian cancer, the combination of paclitaxel with platinum is one of the most active drug combinations. However, most advanced-stage patients relapse and ultimately die of drug resistance. Elevated EGFR expression level has been proven to enhance chemoresistance in ovarian cancer [22]. HB-EGF, which is the only EGFR ligand with particularly enhanced expression in ovarian cancer, may be associated with drug resistance [14]. Heparin-binding epidermal growth factor-like growth factor (HB-EGF) has been proven to be a promising target for ovarian cancer therapy. CRM197, as a specific inhibitor of HB-EGF, may represent a possible chemotherapeutic and chemosensitizing agent for ovarian cancer. The combination of paclitaxel with CRM197 has the synergistic anti-tumor effect on ovarian cancer cell line SKOV3 [19]. However, the expression levels of HB-EGF and the effect of CRM197 treatment on drug-resistant ovarian carcinoma have not been sufficiently elucidated.

In this study, we examined HB-EGF expression in drug-resistant ovarian cancer using drug-sensitive ovarian cancer as the control *in vivo* and *in vitro*. We observed significant elevated HB-EGF expression in A2780/CDDP and A2780/Taxol cells in contrast to A2780 cells. We also found enhanced expression of HB-EGF in xenograft mouse models established with A2780/CDDP and A2780/Taxol cells compared with A2780 tumors. These results indicate that HB-EGF is over-expressed in paclitaxel- and cisplatin-resistant ovarian cancer, suggesting that HB-EGF may play a role in chemoresistant ovarian cancer.

To test the possible significance of the HB-EGF over-expression in A2780/CDDP and A2780/Taxol cells, we inhibited HB-EGF expression by CRM197 to investigate the effect of CRM197 treatment using A2780 cells as a control. This study indicated that CRM197 not only potently suppressed proliferation in a dose-dependent manner, but also induced G0/G1 cell-cycle arrest and cell apoptosis in A2780/Taxol and A2780/CDDP cells. Moreover, enhanced tumorigenicity was observed in xenograft models with

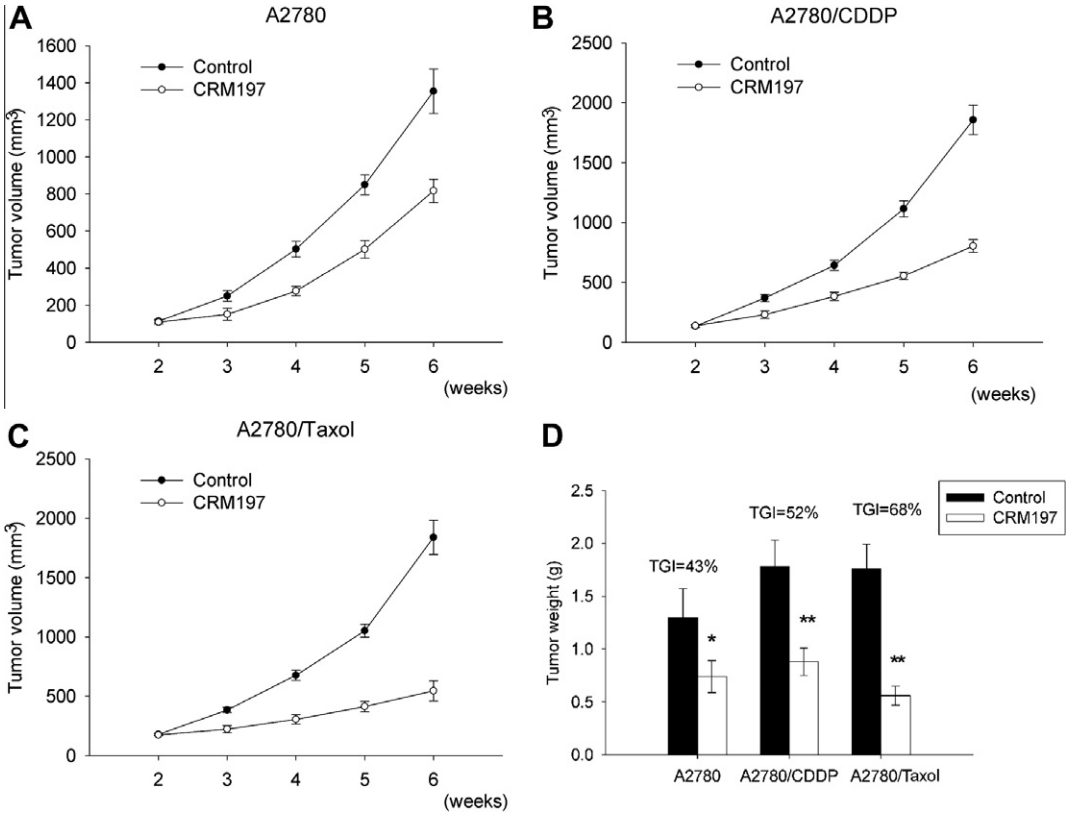

Fig. 3. CRM197 suppressed the growth of drug-resistant ovarian cancer xenografts *in vivo*. $N = 5$ for both the CRM197 group and the control group (tumors treated with PBS). (A–C) Beginning 2 weeks after the subcutaneous injection of A2780, A2780/CDDP and A2780/Taxol cells, tumor size was monitored and measured each week. Tumor volume was calculated by using the equation $x^2y/2$ (where $x < y$). (D) Tumor weight was measured at the completion of the experiment. The values represent the mean ± SD. TGI: tumor growth inhibition. *, $p = 0.004$, **, $p < 0.001$, versus the control group.

A2780/CDDP and A2780/Taxol cells, whereas CRM197 effectively suppressed tumor growth *in vivo*. These results show that HB-EGF plays a key role in tumor formation of resistant ovarian cancer. CRM197 may be a promising therapeutic agent by targeting HB-EGF mediated EGFR signaling and benefit chemoresistant ovarian cancer patients with over-expression of HB-EGF. Intensified research efforts are needed to further clarify this mechanism.

In summary, our findings demonstrate that CRM197, a specific HB-EGF inhibitor, has potent anti-tumor activity in paclitaxel- and cisplatin-resistant ovarian cancer which over-express HB-EGF. CRM197 as an HB-EGF-targeted agent will shed light on the chemotherapy options for future chemoresistant ovarian cancer patients.

5. Competing interest

The authors have declared that no competing interests exist.

6. Funding

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